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JC900 U.S. PTO 08/04/00

UTILITY PATENT APPLICATION TRANSMITTAL
(Under 37 C.F.R. §1.53(b))

Docket No.: 235.00 Page 1 of 2

Title: Factor X Analog With An Improved Ability to Be Activated

Express Mail Label No.: EL524823787US

JC869 U.S. PTO 09/632742 08/04/00

APPLICATION ELEMENTS

ADDRESS TO: Assistant Commissioner for Patents
Box Patent Application
Washington, D.C. 20231

INVENTORS: Michele Himmelspach, Uwe Schlokat

[X] Fee Transmittal Form (Submit an original, and a duplicate for fee processing)

2. [X] Specification [Total Pages [40]]

3. [X] Drawing(s) (35 USC §113) [Total Pages [6]] (Figs.1-5)

4. [ ] Signed Declaration [Total Pages [ ]]

a. [ ] Newly executed (original or copy)

b. [ ] Copy from a prior application (37 CFR §1.63(d))

(Note Box 5 below)

i. [ ] DELETION OF INVENTOR(S)

Signed statement attached deleting invento named in the prior application, see 37 CFR §§1.63(d)(2) and 1.33(b)

5. [ ] Incorporation By Reference (useable if Box 4b is checked.) entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein

6. [ ] Microfiche Computer Program

7. [ ] Nucleotide and/or Amino Acid Sequence Submission

8. [ ] Assignment Papers (cover sheet & document(s))

9. [ ] 37 CFR §3.73(b) Statement [ ] Power of Attorney

10. [ ] English Translation Document

11. [ ] Information Disclosure Statement (IDS)/PTO-1449 [ ] Copies of IDS Citations

12. [ ] Preliminary Amendment

13. [X] Return Receipt Postcard (MPEP 503)

14. [ ] Small Entity Statement(s)

15. [ ] Certified Copy of Priority Documents (s)

16a. [ ] Cancel in the application original claims _____ before calculating the filing fee

16b. [ ] Amend the specification by inserting before the first line the sentence: --This is a ___ Continuation, ___ Divisional, ___ Continuation-in-part, of application ____/_____, filed ____/____/____/

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16d. [ ] Other_____

16e. [ ] Other_____

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UTILITY PATENT APPLICATION TRANSMITTAL (Under 37 C.F.R. §1.53(b))	Docket No.: 235.00	Page 2 of 2
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CLAIMS	(1) FOR	(2) NUMBER	(3) NUMBER EXTRA	(4) RATE	(5) CALCULATIONS
	TOTAL CLAIMS (37 CFR 1.16(b))	43-20 =	23	X $ 18 =	$774
	INDEPENDENT CLAIMS (37 CFR 1.16(b))	3	0	$ 78 =	$0
	MULTIPLE DEPENDENT CLAIMS (if applicable) (37 CFR §1.16(d))			+ $ 260 =	$ 0
				BASIC FEE (37 CFR §1.16(a))	$690
				TOTAL =	$1464

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a. [X] Fees required under 37 CFR §1.16

b. [X] Fees required under 37 CFR §1.17

c. [X] Fees required under 37 CFR §1.18

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

NAME	Janice Guthrie, Reg. No. 35,170
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DATE	**August 4, 2000**

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By: 
Diane M. Branham

FACTOR X ANALOG WITH AN IMPROVED ABILITY TO BE ACTIVATED

Field of the Invention

The present invention relates to factor X analogs with an enhanced ability to be activated by means of a substitution in the region of the activation peptide, a preparation containing the factor X analogs according to the present invention, and a method for the production of single-chain and double-chain factor X analogs.

Background of the Invention

Once the blood coagulation process has been initiated, the coagulation cascade goes through the stages of sequentially activating various proenzymes (zymogens) in the blood into their active forms, the serine proteases. This includes, among others, factor XII/XIIa, factor XI/XIa, factor IX/IXa, factor X/Xa, factor VII/VIIa, and prothrombin/thrombin. Most of these enzymes are active in the physiological state only if they are associated in a complex on a membrane surface. Ca ions are involved in many of these processes. Blood coagulation follows either the intrinsic pathway, in which case all protein components are present in the blood, or the extrinsic pathway, in which the cell membrane tissue factor plays a critical role. Closure of the wound finally takes place as a result of the conversion of fibrinogen into fibrin through the action of thrombin.

The prothrombinase complex is responsible for activating prothrombin to form thrombin. Thrombin is an important enzyme which can act both as a procoagulant and as an anticoagulant. The prothrombinase complex, in which, among others, factor Va (as a cofactor) and factor Xa (as serine protease) participate, assembles in a Ca-dependent association on the surface of phospholipids. It is hypothesized that the catalytic component of the prothrombinase complex is factor Xa.

Factor X (also called Stuart-Prower factor or Prower factor) is a vitamin K-dependent coagulation glycoprotein which participates in the intrinsic and extrinsic blood coagulation cascade. The primary translation product of factor X (pre-pro-FX) contains 488 amino acids and is synthesized by the liver of by human hepatoma cells first as a single-chain 75 kD precursor protein. In the plasma, factor

X is present mainly as a double-chain molecule (Fair et al., Blood 64 (1984), pp. 194-204).

During the biosynthesis, after cleavage of the presequence by a signal peptidase (between Ser23 and Leu24) and the propeptide (between Arg40 and Ala41), the single-chain factor X molecule is cleaved by processing and deletion of the tripeptide Arg180-Lys181-Arg182 into the double-chain form which comprises an approximately 22 kD light chain and an approximately 50 kD heavy chain, the two chains being connected by way of a disulfide bridge (Figure 1). Factor X therefore circulates in the plasma as a double-chain molecule.

During the blood coagulation process, factor X is converted from the inactive zymogen into active protease factor Xa through limited proteolytic action, in the course of which the activation of factor X to form Factor Xa can take place in one of 2 membrane-bound complexes: the extrinsic factor VIIa/tissue factor complex or the intrinsic factor VIIIa/factor IXa phospholipid Ca complex, the so-called "tenase complex" (Mertens et al., Biochem. J. 185 (1980), pp. 647-658). A proteolytic cleavage between amino acids Arg234 and Ile235 leads to the release of a 52 amino acids long activation peptide from the N-terminus of the heavy chain and thus to the formation of the active enzyme, factor Xa. The catalytic center of factor Xa is located on the heavy chain.

The activation via the factor VIIa-TF (extrinsic) complex leads to the formation of factor Xaα (35 kD) and factor Xaß (31 kD), and, if the concentrations of factor VIIa in the complex are low, a polypeptide of 42 kD is present as well.

The formation of factor Xaα takes place via a cleavage at Arg234/Ile 235 of the heavy chain and represents the activation of factor X to form factor Xa. The presence of factor Xaß presumably results from an autocatalytic cleavage at Arg469/Gly470 in the C-terminus of the heavy chain of factor Xaα and the cleavage of a 4.5 kD peptide. Like factor Xaα, factor Xaß also has catalytic activity. It was shown, however, that during the cleavage of factor Xaα to form Xaß, a plasminogen receptor binding site forms and that factor Xaß may also have fibrinolytic activity and may participate as a cofactor in the fibrinolysis. The conversion of factor Xaα into factor Xaß, however, proceeds more slowly than the formation of thrombin, as a result of which the initiation of the fibrinolysis prior to

the formation of a blood clot is prevented (Pryzdial et al., J. Biol. Chem. 271 (1996), pp. 16614-16620; Pryzdial et al., J. Biol. Chem. 271 (1996), pp. 16621-16626).

The 42 kD polypeptide results from a processing in the C terminus of the heavy chain between Arg426 and Gly470 without prior processing between Arg234 and Ile 235. Like a factor Xaγ fragment, this intermediate which forms as a result of proteolysis at Lys370 also does not have any catalytic activity (Mertens et al., Biochem. J. 185 (1980), pp. 647-658; Pryzdial et al., J. Biol. Chem. 271 (1996), pp. 16614-16620).

The activation of factor X in the intrinsic pathway is catalyzed by the factor IXa-factor VIIIa complex. During the activation, the same processing products are obtained, but the factor Xaß product is obtained in a greater yield than other factor X processing products (Jesty et al., J. Biol. Chem. 249 (1974), p. 5614).

In vitro, factor X can be activated, for example, by means of Russell's Viper Venom (RVV) or trypsin (Bajaj et al., J. Biol. Chem. 248, (1973), pp. 7729-7741) or purified physiological activators, such as FVIIa/TF complex or factor IXa/factor VIIIa complex (Mertens et al., Biochem. J. 185 (1980), pp. 647-658).

In most cases, commercially available factor X products from plasma contain a mixture of factor Xaα and factor Xaß since after the activation of factor X to form factor Xa, primarily factor Xaα forms, which, in turn, is cleaved in an autocatalytic process to form factor Xaß.

To produce a uniform factor Xa product with a high molecular integrity, EP 0 651 054 proposed that factor X be activated over a relatively long period of time with RVV, with the result that the resulting final product contained mainly factor Xaß. Both the by-products, for example, factor Xaα, and the protease were subsequently removed in several chromatographic steps.

The cDNA for factor X was isolated and characterized (Leytus et al., Proc. Natl. Acad. Sci. USA 82 (1984), pp. 3699-3702; Fung et al., Proc. Natl. Acad. Sci. USA 82 (1985), pp. 3591-3595). Human factor X was expressed in vitro in various cell types, such as human embryonal kidney cells or CHO cells (Wolf et al., J. Biol. Chem. 266 (1991), pp. 13726-13730). It was found, however, that in the recombinant expression of human factor X, in contrast to the in vivo situation, the

processing in position Arg40/Ala41 is inefficient and that different N termini form on the light chain of factor X (Wolf et al., J. Biol. Chem. 266 (1991), pp. 13726-13730). In vitro, recombinant factor X (rFX) was activated by means of RVV to form recombinant factor Xa (rFXa) or rFXa was directly expressed, in the course of which the activation peptide of amino acid 183 to amino acid 234 was deleted and replaced with a tripeptide to enable processing directly into a double-chain rFXa form. Approximately 70% of the purified rFX were processed to form a light and a heavy chain, and the remaining 30% constituted single-chain rFX with 75 kD. Although the direct expression or rFXa did lead to the formation of active factor Xa, it also led to inactive intermediates. In addition, Wolf et al. (J. Biol. Chem. 266 (1991), pp. 13726-13730) also observed a decreased activity of recombinant factor X, which they attributed to the inferior activation ability of rFX through RVV and to the inactive population of proteins and polypeptides of the single-chain precursor molecule. In particular, they found that rFXa, when expressed by recombinant cells, is highly unstable, which they attributed to the high autoproteolytic rate.

WO 98/38317 describes factor X analogs, in which the amino acids can be modified between Glu228 and Arg234, as a result of which these constructs can be activated, for example, by proteases, such as furin.

To study the function of the C-terminal peptide of factor Xaα, Eby et al. (Blood 80 (Suppl. 1) (1992), pp. 1214 A) introduced a stop codon in position Gly430 of the factor X sequence. They did not, however, find a difference between the activation rate of factor Xa (FXaα) with a ß-peptide and a deletion mutant without a ß-peptide (FXaß).

Factor Xa is an important component of the prothrombinase complex and is therefore used for the quick arrest of bleeding as well as in patients with blood coagulation disorders, such as hemophilia. Especially in the treatment of patients suffering from hemophilia, which is characterized by a factor VIII or a factor IX deficiency, with factor concentrates that are produced from plasma, a complication that frequently arises is that inhibitory antibodies to these factors are formed. Therefore, a number of alternatives were developed to treat patients suffering from hemophilia with factors with a bypass activity. Thus, for example, the use of

prothrombin complex concentrate, partially activated prothrombinase complex (APPC), factor VIIa, or FEIBA has been proposed. Commercial preparations with factor VIII inhibitory bypass activity include, for example, FEIBA® or Autoplex®. FEIBA, for example, contains comparable units of factor II, factor VII, factor IX, factor X, and FEIBA, small quantities of factor VIII and factor V, and traces of activated coagulation factors, such as thrombin and factor Xa and/or a factor with factor Xa-like activity (Elsinger, Activated Prothrombin Complex Concentrates. Eds. Mariani, Russo, Mandelli (1982), pp. 77-87). Elsinger especially stresses the importance of a "factor Xa-like" activity in FEIBA. The factor VIII inhibitory bypass activity was demonstrated by Giles et al. (British J. Hematology 9 (1988), pp. 491-497) in the animal model in particular for a combination of purified factor Xa and phospholipids.

Thus, there is a considerable need and a number of different fields of application for factor X/Xa or factor X/Xa-like proteins, either by themselves or as a component of a coagulation complex in hemostatic therapy. Compared to the half-life of zymogen, the half-life of factor Xa is considerably reduced both in vivo and in vitro. Thus, for example, factor X can be stably stored in glycerol for 18 months while under the same conditions, factor Xa is stable only for 5 months (Bajaj et al., J. Biol. Chem. 248 (1973), pp. 7729-2241), or, if stored in glycerol at 4°C for 8 months, it shows a reduction of the activity by more than 60% (Teng et al., Thrombosis Res. 22 (1981), pp. 213-220). In serum, the half-life of factor Xa is only 30 seconds.

Due to the instability of factor Xa, it has been proposed that factor X preparations be administered (U.S. 4,501,731). In cases of life-threatening bleeding, in particular in patients suffering from hemophilia, however, an administration of factor X has no effect since, due to the lack of the functional "tenase complex," it is not possible for an effective activation of factor X into factor Xa to take place in the intrinsic blood coagulation pathway and since an activation by way of the extrinsic pathway often takes place too slowly to have a rapid effect. Furthermore, patients suffering from hemophilia have a sufficient supply of factor X; however, compared to factor Xa, factor X has a prothrombinase activity that is 1000 times lower. In cases of this type, activated factor Xa must be administered directly, possibly in

combination with phospholipids, such as described by Giles et al. (British J. Haematology 9 (1988). pp. 491-497), or with other coagulation factors, for example, with factor VIII inhibitory bypass activity.

In the production of factor Xa from factor X, the activation has so far been triggered mainly by means of nonphysiological activators of animal origin, such as RVV or trypsin, but this means that care has to be taken to ensure with absolute certainty that the final product is completely free from these proteases. As already mentioned above, during the activation of factor X to factor Xa, a large number of inactive intermediates is formed (Bajaj et al., J. Biol. Chem. 248 (1973), pp. 7729-7741, Mertens et al., Biochem. J. 185 (1980), pp. 647-658). The presence of such intermediates leads to a decrease of the specific activity of the product and potentially even to the type of intermediates that might serve as antagonists of the active serine protease. Thus, to produce a uniform, pure product with a high specific activity by means of conventional methods, time-consuming and complicated procedures for the activation and chromatographic purification are required.

Summary of the Invention

The present invention provides a preparation which contains a polypeptide with factor X/Xa activity, which, compared to prior art, can be more readily activated by factor XIa or a derivative thereof, which has a high stability, and which can be activated by means of factor XIa or a derivative thereof, without having to use one of the proteases used in prior art to activate the natural factor X, particularly one of animal origins, such as RVV or trypsin. Another objective of the present invention is to make available a pharmaceutical preparation with factor VIII inhibitory bypass activity.

Brief Description of the Drawings

Figure 1 presents the nucleotide and amino acid sequence of factor X (Seq.ID NO. 1 and 2).

Figure 2 is a diagrammatic representation of the factor X analog with a modified protease cutting site in the region of the activation peptide.

Figure 3 shows a Western blot analysis of recombinant factor X expressed in CHO cells.

Figure 4 shows a Western blot analysis after the in vitro activation of the factor X analog with factor Xia.

Figure 5 shows the purification of rfX/rFXIa (Q-R/I) by anion exchange chromatography.

Detailed Description of the Invention

The present invention provides a Factor X analog with a modification in the region of amino acid residues 226-235 with reference to the sequence shown in Figure 1

Herein, the term "modification" refers to a mutation, a deletion, an insertion, or a substitution of an amino acid residue within the designated sequence. The term "substitution" refers to replacement of an amino acid residue with a different amino acid residue within the polypeptide. The term "deletion" refers to the absence of at least one of the amino acid residues within the polypeptide, without replacement by another amino acid residue. The term "insertion" refers to the placement of an extra amino acid residue within the polypeptide. The term "mutation" refers to any change in the sequence of the designated polynucleotide or polypeptide, which change could be a deletion, an insertion, or a substitution of one or more nucleic acids or amino acids within the designated nucleic acid or amino acid sequence. Preferably, in the present invention, the modification is a substitution of at least one amino acid.

The amino acid modification in this region creates a new recognition and processing site for factor XIa or a derivative thereof, which site does not naturally occur in this position in the polypeptide. Factor XIa or a derivative thereof does not normally cleave Fx in the region of Glu-Arg-Gly-Asp-Asn-Asp-Phe-Thr-Arg/Ile of amino acids 226-234. Surprisingly, the factor X analog according to the present invention has an at least 2-fold, preferably an at least 5-fold, and especially an at least 10-fold increased ability to be activated by factor XIa compared to the factor X analog according to WO 98/38317.

In addition, it was a surprise to discover that the factor X analog according to the present invention at an antigen concentration of 4-8 µg/mL is able to reduce the coagulation time of factor IX- or FVIII-deficient plasma more effectively than >200 mU, preferably >500 mU, especially >1000 mU of plasma factor IX or FVIII.

Preferably, a minimum of one of the amino acid 226-230, especially 226-228, is modified. It is to be especially preferred if as many of the amino acids in the 226-235 domain as possible were to correspond to a cleavage site for factor XIa or a derivative thereof. According to the present invention, the introduction of a specific factor XIa cleavage sequence which comprises a minimum of 4, preferably a minimum of 6 amino acids, has proven to be especially useful.

The modification is preferably selected to ensure that the processing by means of factor XIa leads to a polypeptide which corresponds to the native factor Xa and which essentially resembles the naturally occurring factor Xa sequence and also has factor Xa activity.

To ensure optimum processing, in some cases, it may be necessary to find a modify the amino acid Ile235. Preferably, however, the NH_2-terminal amino acid isoleucine of the heavy chain should be maintained after the activation since this amino acid plays an important role in the formation of the substrate-binding pocket (Watzke et al.(1995), Molecular Basis of Thrombosis and Hemostasis, eds. Katherine High and Harold Roberts). Compared to the native factor X sequence, the factor X analogs according to the present invention are structurally different, in particular on the amino acid level, but their ability to be activated is comparable to the naturally occurring factor X and, after activation, factor Xa activity.

The invention makes available factor X analogs which are modified in the activation peptide relative to the naturally occurring factor X sequence and which have a changed protease specificity. Amino acid Modifications may take place in position Ile235 (R1), Arg234, Thr233 (R2), Leu 232 (R3), Asn231 (R4), Asn230 (R5), Asp229 (R6), Gly228 (R7), and Arg229 (R8), while Arg234, however, preferably remains unchanged.

The factor X analogs according to the present invention preferably have a factor X sequence with Glu226-R8-R7-R6-R5-R4-R3-R2-Arg234-R1, wherein

a) R1 is an amino acid selected from the group Ile, Val, or Ala;

b) R2 is an amino acid selected from the group Thr, Ser or Asn;

c) R3 is an amino acid selected from the group Phe, Leu, Arg, or Ile;

d) R4 is an amino acid selected from the group Asp, Lys, Thr, or Glu;

e) R5 is an amino acid selected from the group Asn, Ser, Lys, Met, Thr, or Asp;

f) R6 is an amino acid selected from the group Phe, Thr, Ser, Pro, Leu, or Ile;

g) R7 is an amino acid selected from the group Ser, Gln, Ile, Thr, Asn, or Pro; and

h) R8 is an amino acid selected from the group Gln, Ser, His, Tyr, or Glu.

According to the present invention, preferably at least 4 amino acids of amino acids 226-234 differ from the natural factor X sequence, and preferably at least 3 of the amino acid modifications follow one immediately after the other.

Preferably, the amino acid sequences are of the kind that correspond to the amino acid sequences of the activation peptide of factor IX. These sequences can correspond to human, but also to animal (e.g., murine, porcine, etc.) factor IX activation peptide regions.

Embodiments of the factor X analogs according to the present invention to be preferred are FX analogs which are modified as follows:

a) R1 = Val, R2= Thr, R3 = Leu, R4 = Asp, R5 = Asn, R6 = Asp, R7 = Ser, and R8 = Gln and which are processed by means of factor XIa or a derivative thereof;

b) R1 = Ile, R2 = Thr, R3 = Leu, R4 = Asp, R5 = Asn, R6 = Asp, R7 = Ser, and R8 = Gln and which are processed by means of factor XIa or a derivative thereof (Figure 2);

c) R1 = Val, R2 = Thr, R3 = Leu, R4 = Lys, R5 = Ser, R6 = Thr, R7 = Gln, and R8 = Ser and which are processed by means of factor XIa or a derivative thereof; and

d) R1 = Ile, R2 = Thr, T3 = Leu, R4 = Lys, R5 = Ser, R6 = Thr, R7 = Gln, and R8 = Ser and which are processed by means of factor XIa or a derivative thereof.

Other useful embodiments can be obtained by exchanging one or two other amino acids of molecules a)-d) above for which the assays made available according to the present invention have shown that they are cleavable by means of factor XIa.

The modifications can be carried out, for example, by site-specific in vitro mutagenesis or by PCR or by any other genetic engineering methods known in prior art which are suitable for specifically changing a DNA sequence in order to carry out specific amino acid exchanges.

According to the present invention, the activation of the factor X analog according to the present invention to form a native factor Xa or a factor Xa analog is carried out by means of factor XIa or a derivative thereof.

One of the difficulties encountered in the production of active factor Xa is its instability since in addition to factor Xaα, factor Xaß and other intermediates, some possibly inactive, are formed as a result of autocatalysis.

To produce substantially intact active factor X/Xa and/or factor X/Xa-like molecules, it would therefore be desirable to obtain only proteins which lead to stable final products.

It is known that a preferred cleavage site for processing factor Xaα (FXaα) to form factor Xaß (FXaß) is located between Arg469 and Gly470. According to studies by Eby et al. (Blood, Vol. 80, Suppl. 1 (1992), p. 1214), in addition to a prominent carboxy-terminal peptide (amino acid residues 476-487) of factor X, an additional shorter peptide (amino acid residues 474 to 477) is found, which forms as a result of autocatalysis of factor Xaα. To focus on the specific processing of intact factor X to form active factor Xa, without obtaining inactive processing intermediates, the factor X analogs according to the present invention have other modifications.

Thus, according to a special embodiment of this invention, the factor X analog according to the present invention is further modified in the C-terminal region of the factor X amino acid sequence.

According to one embodiment of the present invention, a factor X analog of the type described above has an intact ß-peptide (FXα). Herein, the term "ß-peptide" refers to a 4kD glycopeptide as known from prior art. The first cleavage site for the removal of the ß-peptide by plasmin is located at Arg 469 according to amino acid sequence of Fig. 1, resulting in the removal of the complete C-terminus, i.e. the ß-peptide (Pryzdial E. and Kessler G., J. Biol. Chem., 1996, pp. 16614-16620). In particular, the factor X analog according to the present invention has a

modification in the region of the C-terminal ß-peptide cleavage site which ensures that a cleavage of the ß-peptide from factor X is prevented after factor X has been activated to form factor Xa. This results in a factor Xa molecule, of which up to 100% can be isolated in the form of an intact factor Xaα molecule.

The modification can be a mutation, deletion, or insertion in the region of the factor X amino acid sequence between amino acid positions Arg469 and Ser476 and potentially of Lys370. An amino acid modification to be preferred, however, is one in which it is not possible for a folding of the polypeptide, which would influence the structure and thus possibly the function and activity of the protein, to occur as a result of the amino acid exchange.

According to another embodiment of the present invention, the factor X analogs according to this invention comprise an exchange of one of the amino acid in position Arg469 and/or Gly470, with Arg469 preferably being exchanged for Lys, His, or Ile and with Gly470 preferably being exchanged for Ser, Ala, Val, or Thr.

In addition to a mutation in position Arg469 and/or Gly470, the factor X analogs according to the present invention may have an additional mutation in position Lys370 and/or Lys475 and/or Ser476.

As a result of an amino acid modification in one of these positions, processing of factor Xaα into factor Xaß or factor Xaγ is avoided since the naturally occurring processing sequence(s) is (are) modified to ensure that it is no longer possible for a potential autocatalytic cleavage of the carboxy-terminal peptide to occur.

According to yet another embodiment of the present invention, the factor X analog according to the present invention has a deletion of the carboxy-terminal ß-peptide (FXß). Such a factor X analog can be produced by expressing a cDNA which codes for a factor X analog in a recombinant expression system, with only those sequences being cloned which code for the amino acids Met1 to Arg469.

In yet another embodiment of the present invention, the factor X analog according to this invention has a translation stop signal in the C-terminal region of the factor X sequence. This translation stop signal is preferably in a position which follows a C-terminal amino acid that is formed after natural processing. The

translation stop signal is therefore preferably in the position of amino acid 470 of the factor X sequence so that the terminal Arg469 of factor Xaß is maintained. To ensure this, codon GGC which codes for the amino acid Gly470 is exchanged for TAA, TAG, or TGA.

Another feature of the present invention relates to factor X analogs which are activated by treating them in vivo and in vitro with factor XIa or a derivative thereof to obtain native factor Xa or a factor Xa analog, i.e., the activated factor X analogs. Depending on the factor X analog that is used and activated, one obtains a polypeptide which corresponds to and is essentially identical to the native factor Xa or a polypeptide which, although it has factor Xa activity, has modifications relative to the native factor Xa sequence which, however, do not impair the biological activity. When the factor X analogs according to the present invention, which are modified in the region of the activation peptide in the sequence of the activation peptide, are activated, only polypeptides which correspond to the native factor Xa molecule are obtained. If such a factor X analog also has an additional translation stop signal in the C-terminal region of the ß-peptide, molecules homologous to factor Xaß are obtained. If, on the other hand, a factor X analog is used, which has a modification or modifications within the ß-peptide sequence which has or have the effect that that ß-peptide is not cleaved off, a factor Xaα analog with an amino acid exchange in the C-terminus of the molecule is obtained.

The factor X analogs according to the present invention have only modifications which change the specificity for the activation ability and which do not have a negative effect on the activity. Therefore, in all cases, biologically and functionally active factor Xa molecules and factor Xa analogs are obtained.

The activation in vivo and in vitro can be carried out by means of factor XIa or a derivative thereof. In this context, a factor XIa derivative can be a polypeptide or protein derived from factor XIa which differs from the native factor XIa, for example, with respect to its length (e.g., truncated forms) or which has been obtained by amino acid modification. In all cases, it is important to ensure that the factor XIa derivative also has the specific protease activity that is characteristic for factor XIa.

According to a special embodiment, the present invention makes available factor X analogs which are preferably present in purified form as single-chain molecules. The single-chain factor X molecule is marked by a high stability and molecular integrity. Up to now, it had not been possible to isolate a single-chain factor X molecule in purified form since it is very rapidly processed into the double-chain form (Fair et al., Blood 64 (1984), pp. 194-204). The recombinant single-chain factor X analogs can be processed by specific processing to form the double-chain factor X form and can be subsequently activated into factor Xa or the factor Xa analog. This can be accomplished by having the single-chain factor X analog come into contact with furin, by processing it, and by subsequently activating it by means of factor XIa or a derivative thereof.

The double-chain factor X analog can be activated to form factor Xa or a factor Xa analog. This can be accomplished, for example, by using a factor X analog which, as a result of the mdification according to the present invention in the region of the activation peptide, has a factor XIa cleavage site, which is expressed and isolated in a recombinant cell as a single-chain molecule, and which is subsequently processed by bringing it into contact with furin and then cleaved by means of factor XIa or a derivative thereof to form an activated factor Xa molecule.

A factor X analog which was isolated as a double-chain molecule from a cell culture can be treated directly with factor XIa or a derivative thereof.

Due to the selective and site-specific processing reaction, a factor Xa or a factor Xa analog obtained in this manner has a high stability and structural integrity and, in particular, is free from inactive factor X/Xa analog intermediates and autoproteolytic degradation products. In addition, the factor X analog according to the present invention can be especially readily activated by means of factor XIa or a derivative thereof, with the ability to be activated being at least 2-fold, preferably at least 5-fold, and especially at least 10-fold increased when compared to the factor X analogs described in WO 98/38317. Surprisingly, it was discovered that the constructs according to the present invention can be more readily activated as a result of the fact that a minimum of 4, preferably a minimum of 6 of amino acid 226-235 are amino acids that differ from those in the natural factor X molecule and

that preferably at least 3 of the exchanged amino acid follow one immediately after the other.

An additional feature of the present invention relates to the recombinant DNA which codes for the factor X analogs according to the present invention. After its expression, the recombinant DNA results in a suitable host cell in a factor X analog with an amino acid sequence that corresponds to human factor X, except that it has an amino acid modification that influences the processing specificity and the processing products. The biological coagulation activity, however, is not in any way negatively influenced; instead, surprisingly, frequently even an increase in the activity results.

According to yet another feature of the present invention, transformed cells containing the recombinant DNA are also made available.

An additional feature of the present invention relates to a preparation containing a purified factor X analog or a precursor protein thereof which has the amino acid modification according to this invention in the region of the naturally occurring factor Xa activation site. The modification in the region of the activation cleavage site is a new recognition and processing cleavage site -- which does not naturally occur in this position in the polypeptide -- for factor XIa or a derivative thereof which does not normally process the polypeptide in this position. The preparation can be a purified preparation of factor X analogs, with the polypeptides being obtained from a cell culture system either after isolation from the supernatant of the cell culture or from an extract of a cell culture. A prepurified recombinant factor X analog from a cell culture system can be further purified using procedures known from prior art. In this context, chromatographic processes, such as gel filtration, ion-exchange or affinity chromatography, are particularly suitable for use.

According to one embodiment of the invention, the preparation according to this invention preferably contains the factor X analog as a single-chain molecule in isolated form. Such a preparation is produced by isolating a factor X analog, which was obtained by recombinant production, as a single-chain molecule from a cell system, preferably from a cell culture of cells deficient in endoprotease.

A special feature of the present invention relates to the fact that the preparation contains a single-chain factor X analog with a modification which, after

processing by means of furin, allows an in vitro activation into factor Xa by means of factor XIa or a derivative thereof. The activation is accomplished by bringing the factor X analog into contact with the proteases, which leads to a cleavage into the mature factor X form and, as a result of the modification, to a cleavage of the activation peptide and to the formation of factor Xa and the factor Xa analog.

In the preparation according to the present invention, the factor X analog can be present either as factor Xα (FXα) or with a deletion of the ß-peptide.

The preparation contains, in particular, a factor X analog in an enzymatically inactive form with a purity of a minimum of 80%, preferably 90%, and especially 95% and does not contain any inactive proteolytic intermediates of the factor X/Xa analog.

According to another embodiment of the present invention, the preparation according to this invention preferably contains the factor X analog as a double-chain molecule in isolated form. To accomplish this, for example, a factor X analog which has been obtained by means of recombinant production as a single-chain molecule from a cell system is cleaved in vitro, i.e., outside the cell, by means of furin to obtain the double-chain form. This can be accomplished by mixing the protease directly with the supernatant of the culture of the clones that express the factor X analog, either by mixing the purified protease or a cell culture supernatant of a cell culture which expresses the protease in recombinant form or by means of co-cultivation of factor X analog- and protease-expressing clones.

According to a special embodiment of the present invention, the preparation containing the purified, single-chain or double-chain factor X analog contains a physiologically acceptable matrix and is potentially formulated as a pharmaceutical preparation. The preparation can be formulated using substantially known prior art methods, it can be mixed with a buffer containing salts, such as NaCl, $CaCl_2$, and amino acids, such as glycine and/or lysine, in a pH range from 6 to 8, and it can be formulated as a pharmaceutical preparation. Until needed, the purified preparation containing the factor X analog can be stored in the form of a finished solution or in lyophilized or deep-frozen form. Preferably the preparation is stored in lyophilized form and is dissolved into a visually clear solution using an appropriate reconstitution solution.

But the preparation according to the present invention can also be made available as a liquid preparation or as a liquid that is deep-frozen.

The preparation according to the present invention is especially stable, i.e., it can be allowed to stand in dissolved form for a prolonged time prior to application. It was found that the preparation according to this invention can be allowed to stand for several hours and even days without loss of activity.

The preparation according to the present invention can be placed into a suitable device, preferably an application device, in combination with factor XIa or a derivative thereof.

The preparation according to the present invention which contains a factor X analog in combination with factor XIa or a derivative thereof which is able to activate the factor X analog into factor Xa or the factor Xa analog can be made available in the form of a combination preparation comprising a container that holds factor XIa which is immobilized on a matrix, potentially in the form of a miniature column or a syringe complemented with a protease, and a container containing the pharmaceutical preparation with the factor X analog. To activate the factor X analog, the factor X analog-containing solution, for example, can be pressed over the immobilized protease. During storage of the preparation, the factor X analog-containing solution is preferably spatially separated from the protease. The preparation according to the present invention can be stored in the same container as the protease, but the components are spatially separated by an impermeable partition which can be easily removed before administration of the preparation. The solutions can also be stored in separate containers and be brought into contact with each other only shortly prior to administration.

The factor X analog can be activated into factor Xa shortly before immediate use, i.e., prior to the administration to the patient. The activation can be carried out by bringing a factor X analog into contact with an immobilized protease or by mixing solutions containing a protease, on the one hand, and the factor X analog, on the other hand. Thus, it is possible to separately maintain the two components in solution and to mix them by means of a suitable infusion device in which the components come into contact with each other as they pass through the device and thereby to cause an activation into factor Xa or into the factor Xa analog.

The patient thus receives a mixture of factor Xa and, in addition, a serine protease which is responsible for the activation. In this context, it is especially important to pay close attention to the dosage since the additional administration of a serine protease also activates endogenous factor X, which may shorten the coagulation time.

According to a useful embodiment of the invention, the pharmaceutical preparation is made available in a suitable device, preferably an application device, either in the form of a frozen liquid or in freeze-dried form. A suitable application device, for example, is a double-compartment syringe of the type described in the AT 366 916 or AT 382 783.

One especially useful feature of this invention is that the preparation can contain a factor X analog with a modification which makes possible an in vivo activation of the factor X analog into factor Xa. In particular, the factor X analogs of the preparation according to the present invention have a modification which represents a recognition and cleavage site for factor XIa or a derivative thereof, thus making it possible for them to be cleaved by this protease in vivo to form native factor Xa or the factor Xa analog. As a result, the preparation according to the present invention can be used to arrest bleeding both in patients with deficiencies of factor IX and factor VIII and in patients with factor VIII inhibitor.

The preparation according to the present invention can be made available as a pharmaceutical preparation with factor Xa activity in the form of a one-component preparation or in combination with other factors in the form of a multi-component preparation.

Prior to processing the purified protein into a pharmaceutical preparation, the purified protein is subjected to the conventional quality controls and fashioned into a therapeutic form of presentation. In particular, during the recombinant manufacture, the purified preparation is tested for the absence of cellular nucleic acids as well as nucleic acids that are derived from the expression vector, preferably using a method, such as is described in EP 0 714 987.

Since any biological material can be contaminated with infectious microorganisms, the preparation may have to be especially treated to inactivate or deplete viruses in order to ensure that a safe preparation is obtained.

Another feature of this invention relates to making available a preparation which contains a factor Xa analog with a high stability and structural integrity and which, in particular, is free from inactive factor X/Xa analog intermediates and autoproteolytic degradation products and which can be produced by activating a factor X analog of the type described above and by formulating it into an appropriate preparation.

An additional feature of the present invention relates to the use of a preparation of the type described above to produce a drug. A drug which contains a factor X analog and/or a factor Xa analog according to the present invention is suitable especially for the treatment of patients with blood coagulation disorders, such as patients suffering from hemophilia or hemophiliacs with inhibitory antibodies, and, in particular, as a preparation with factor VIII inhibitor bypass activity.

Another feature of this invention relates to the use of a nucleic acid which contains the coding sequences of the factor X analog according to this invention for the production of a drug. In this context, the nucleic acid, to the extent that it contains suitable expression control sequences, can be administered in the form of naked nucleic acid, it can be integrated into a recombinant expression vector, or it can be bound to a matrix, such as either a phospholipid or a viral particle. The nucleic acid can be used to manufacture a drug which is especially suitable for use in the treatment of patients with blood coagulation disorders, such as hemophilia patients or hemophiliacs with inhibitory antibodies. Another potential application is the use of the nucleic acid in gene therapy.

Another feature of the present invention relates to a process for the production of the factor X analogs according to this invention and a preparation containing a factor X analog according to this invention. To accomplish this, a sequence coding for a factor X analog is introduced into a suitable expression system and the relevant cells, preferably permanent cell lines, are transfixed with the recombinant DNA. The cells are cultivated under optimum conditions for gene expression, and factor X analogs are either isolated from the extract of a cell culture or from the supernatant of the cell culture. The purification of the recombinant molecule can be further carried out using all conventionally known

chromatographic procedures, such as anion- or cation-exchange chromatography, affinity or immunoaffinity chromatography or a combination thereof.

To produce the factor X analogs according to the present invention, the complete cDNA coding for factor X is cloned into an expression vector. This is carried out by means of the generally known cloning techniques. The nucleotide sequence coding for factor X is subsequently modified to ensure that the coding sequence in the region of the activation peptide and possibly in the region of the C-terminal ß-peptide is changed to ensure that a factor X molecule of the type described above can be produced. To accomplish this, genetic engineering methods known from prior art are used, for example, site-specific in vitro mutagenesis, or deletion of sequences, for example, by means of digestion through restriction by endonucleases and insertion of different, changed sequences, or by PCR. The factor X mutants thus produced are then inserted into an expression system suitable for the recombinant expression and are subsequently expressed.

The factor X analogs according to the present invention can also be produced by means of chemical synthesis.

The factor X analogs are preferably produced by means of recombinant expression. The production by means of genetic engineering methods can be carried out with all known expression systems, e.g., permanent cell lines or viral expression systems. The permanent cell lines are produced by stable integration of exogenous DNA into the host cell chromosome, e.g., of Vero, MRC5, CHO, BHK, 293, Sk-Hep1, especially liver and kidney cells, or by an episomal vector derived, e.g., from papillomavirus. Viral expression systems, such as vaccinia virus, baculovirus, or retroviral systems can also be used. The cell lines normally used are Vero, MRC5, CHO, BHK, 293, Sk-Hep1, glandular, liver, and kidney cells. Eukaryotic expression systems to be used include yeasts, endogenous glands (e.g., glands of transgenic animals) and other cell types. It is, of course, also possible to use transgenic animals to express the polypeptides according to the present invention or derivatives thereof. To express the recombinant proteins, especially CHO-DHFR cells have been shown to be useful (Urlaub et al., Proc. Natl. Acad. Sci. USA 77, (1980), pp. 4216-4220).

For the recombinant production of the factor X analogs according to the present invention, it is also possible to use prokaryotic expression systems. Especially suitable are systems which allow an expression in E. coli or B. subtilis.

The factor X analogs are expressed in the appropriate expression systems using a suitable promoter. For the expression in eukaryotes, all known promoters, such as SV40, CMV, RSV, HSV, EBV, ß-actin, hGH, or inducible promoters, such as hsp or metallothionein promoter, can be used. The factor X analogs are preferably expressed using the ß-actin promoter in CHO-DHFR cells.

According to one embodiment of the invention, the method for producing the preparation according to the present invention comprises the following steps: preparing a DNA which codes for a factor X analog, transformation of a cell with the recombinant DNA, expression of the factor X analog, potentially in the presence of a protease, isolation of the factor X analog, and potential purification by means of a chromatographic procedure.

According to one embodiment of the method, the factor X analog is isolated as a double-chain molecule.

For this purpose, the factor X analog is expressed in a cell which allows processing of profactor X analogs in double-chain factor X analogs.

The double-chain factor X analog thus obtained can subsequently be isolated, purified, and, as described above, stably stored until further use.

According to one embodiment of this invention, the activation is triggered by a chromatographic step in which the protease is immobilized on a matrix. Purified double-chain factor X analog is passed over a matrix to which the protease is bound, and from the eluate, purified factor Xa is isolated.

According to another embodiment of the invention, the components are mixed and the protease is selectively removed from the mixture.

In addition, it is, of course, also possible to process a single-chain profactor X analog into the double-chain factor X analog form and activate it into factor Xa in a single process.

The reaction conditions of processing reaction(s) and the activation can be readily optimized by those skilled in the art depending on the experimental setup of any given situation. In this context, it should be noted that the flow rate of the

reaction participants used is of special importance to the length of contact time. This flow rate should be in a range from 0.01 mL/min to 1 mL/min. Other important parameters are the temperature, the pH value, and the elution conditions. At the end of the flow time, the activated factor Xa can optionally be further purified by means of selective chromatography. Carrying out the method with protease that is bound to a matrix offers a special advantage since, as a result of the use of a matrix, preferably of chromatographic columns, the reaction setup makes it possible to include an additional purification step.

An additional feature of the production of a factor X analog according to this invention is that the factor X analog is isolated as a single-chain molecule. For this purpose, the factor X analog is expressed in a cell in which the cleavage of the light and heavy chain of factor X and/or a factor X analog cannot take place. Furin is of the important proteases that are responsible for the cleavage of factor X into a light and a heavy chain. From such an endoprotease-deficient mutant cell, it is possible to isolate the factor X analog in the form of a single-chain molecule. A factor X analog that was isolated in this manner and potentially also purified is subsequently brought into contact with furin under conditions under which the single-chain factor X analog is cleaved into the double-chain factor X form. Factor X analogs according to the present invention which have a modification in the region of the activation peptide that makes a cleavage by factor XIa possible can subsequently be activated by this method, possibly directly by bringing them into contact with factor XIa or a derivative thereof, to form factor Xa or the factor Xa analog.

According to another feature of this invention, using the method according to the invention, a preparation containing the active factor Xa or an active factor Xa analog is obtained by subjecting a factor X analog that had been obtained as described above to an activation step and by processing the activated polypeptide into a purified preparation which is potentially formulated as a pharmaceutical compound.

Using the factor X analogs according to the invention which are activated by a process described above into factor Xa, a purified factor Xa and/or factor Xa

analog with a high stability and structural integrity is obtained which, in particular, is free from inactive factor X/Xa intermediates.

This invention will be explained in greater detail on the basis of the following examples and figures shown in the drawing, without, however, thereby limiting the invention in any way.

Examples:

Example 1:

Construction and expression of recombinant factor X wild type (rFX) and factor X/FXIa (Q-R/I) analogs

a. Production of the rFX expression vector phAct-rFX

The cDNA of FX was isolated from a human liver lambda-cDNA bank as described by Messier et al. (Gene 99 (1991), pp. 291-294). By means of PCR, using oligonucleotide #2911 (5'-ATTACTCGAGAAGCTTACCATGGGGCGCCCACTG-3') (SEQ ID No. 3) as the 5' primer and oligonucleotide #2912 (5'-ATTACAATTGCTGCAGGGATCCAC-3') (SEQ.ID No. 4) as the 3' primer, a DNA fragment was amplified from a positive clone, which DNA fragment contains the 1,457 kB FX-coding sequence and 39 bp of the 3' nontranslated region, flanked by an XhoI cutting site on the 5' end and an MfeI cutting site on the 3' end. In addition, by means of primer #2911, the sequence ACC was inserted in front of ATG of FX, thus ensuring that an optimum Kozak translation initiation sequence forms. Subsequently this PCR fragment was cloned as XhoI/MfeI fragment into the expression vector phAct which had been cut with SalI an EcoRI. The expression factor phAct comprises approximately 3.3 kb of the promoter, 78 bp of 5' UTR, and the approximately 1 kb measuring intron of the human beta-actin gene (Fischer et al., FEBS Lett. 351 (1994), pp. 345-348), a multiple cloning cutting site and the SV40 polyadenylation site. The resulting expression plasmid was called phAct-rFX.

b. Production of the phAct-rFX/FXIa (Q-R/I) expression plasmid

To produce recombinant FX/FXIa (Q-R/I) analogs, the amino acid sequence from position 227 to 234 (Arg-Gly-Asp-Asn-Asn-Leu-Thr-Arg/Ile) which serves to

activate FX into FXa was replaced with the intrinsic tenase complex FIXa/FVIIIa or by the extrinsic FVIIa/TF complex, by the sequence Gln-Ser-Phe-Asn-Asp-Phe-Thr-Arg/Ile (hereinafter referred to as (Q-R/I)), specifically activated by means of the coagulation factor XIa (Figure 2). Q-R was prepared in conformity with the second FXIa cutting site, such as it is present in the 'natural' substrate FIX. The expression plasmid for this rFX analog is derived from plasmid phAct/rFX. For cloning purposes, the HindIII-NaeI DNA fragment from the phAct-rFX expression plasmid which comprises the FX-coding region from position +1 to +1116 was inserted into the HindIII-SmaI restriction cutting sites of plasmid pUC19. The resulting plasmid was called pUC/FX. This made it possible for the FX sequence of nucleotide 508 to 705 which corresponds to amino acids 160 to 235 to be removed from the pUC/FX plasmid via Bsp120I and BstXI restriction cuts and to be replaced by a mutated FX-DNA fragment. The mutated DNA fragment contains the FX-exogenic cleavage site for FXIa, instead of the FIXa/FVIIIa and FVIIa/TF site, and is produced by means of PCR. Plasmid phAct/rFX serves as a model for the PCR. To produce the Gln-Ser-Phe-Asn-Asp-Phe-Thr-Arg/Ile (Q-R/I) cleavage site, oligonucleotide #4211 (5'-GGCAAGGCCTGCATTCCCACA-3') (SEQ ID. No. 5) is used as the 5' primer and oligonucleotide #5039 (5'-GCGCTCCCACGATCCTGGTGAAGTCATTAAAGCTTTGCTCAGGCTGC GTCTGGTT-3') (SEQ ID No. 6) is used as the 3' primer. Therefore, amino acids Arg, Gly, Asp, Asn, and Leu are replaced in positions 227, 228, 229, 231, and 232 with Gln, Ser, Phe, Asp, and Phe. The PCR product is recut with Bsp120I and BstXI and inserted into the pUC-FX plasmid that had been opened with Bsp120I/BstXI. Subsequently, the DNA fragment which contains the new cleavage site is reinserted via HindIII/AgeI into phact-FX which had been cut with the same restriction enzymes. The resulting plasmid is called phAct-FX/FXIa (Q-R/I).

c. Expression of rFX and of the rFX/FXIa (Q-R/I) analog in CHO cells

To establish stable rFX/FXIa-expressing cell lines, the expression plasmid phAct-rFX and phAct-rFX/FXa (Q-R/I) is co-transfixed with the selection marker pSV-dhfr in the dhfr-deficient CHO cells (Fischer et al., FEBs Lett. 351 (1994), pp.

345-348). For all other expression and function analyses, the cell cultures are incubated after a complete media change with a serum-free selection medium in the presence of 10 µg/mL vitamin K for 24 h. The expression of rFX in the resulting cell clones is demonstrated on the basis of the quantity of antigen (ELISA, Asserachrom, Boehringer Mannheim), and the recombinant protein is subsequently characterized with SDS-PAFW (Figure 3). As can be seen in the Western blot as well, both the rFX wild type and the rFX/FXIa (Q-R/I) molecules are present in the form of a light chain (LC) of approximately 22 kD and a heavy chain (HC) of approximately 50 kD which correspond to the plasmatic factor X forms. In addition, a 75 kD protein band can be seen. The 75 kD protein corresponds to the single-chain (SC) FX molecule, the presence of which had already been described in the supernatants of FX-transfixed CHO cells (Wolf et al., J. Biol. Chem. 266 (1991), pp. 13729-13730) and in human plasma (Fair et al., Blood 64 (1984), pp. 194-204).

Example 2:

In-vitro activation of the rFX/FXIa (Q-R/I) molecules by means of factor XIa

To demonstrate the cleavability of the newly inserted activation sites by means of factor XIa, the supernatants of the cell culture are mixed with purified factor XIa in the presence of 5 mM $CaCl_2$. Aliquot portions of the reaction batches are tested for cleavage both prior to the incubation and after different incubation times at 37°C by means of the Western blot analysis (Figure 4). The identification of the rFX molecules is carried out by means of a polyclonal anti-human FX antibody. The positive control used is purified plasma factor IX (Stago) which is the natural substrate for FXIa. To work under uniform conditions, FIX is diluted prior to use in supernatants of the cell culture of nontransfixed CHO cells. The conversion of FIX into the activated forms (FIXa and FIXb) shows that FXIa is able to cleave its homologous cutting site in FIX in supernatants of the cell culture. The reaction batch with rFX wild type serves as a negative control since this molecule does not contain any FXIa cleavage site and should therefore not be specifically recognized and cleaved by FXIa. As expected, compared to rFX in the absence of FXIa, no change for rFX is visible in the protein pattern after incubation with FXIa.

In contrast, after an incubation time of 1 h at 37°C in the presence of $CaCl_2$, the reaction batch of FXIa, with rFX/FXIa (Q-R/I) as a substrate, shows protein bands of approximately 36 kD and 32 kD which resemble the activated alpha and beta forms (aHCa and bHCa) of the heavy chain of FX. In the absence of FXIa, these cleaved molecules are not present. The presence of a form which is similar to bHCa and which forms as a result of the autocatalytic cleavage of C-terminal amino acids of the heavy chain of aHCa points to the functionality of the generated activated rFXa molecules. These results show that by replacing the naturally existing activation sequence in FX with an activation cleavage site for FXIa, it is possible to generate rFX analog molecules which can be activated by FXIa and which can subsequently be converted into molecules that resemble FXa.

The method described can be used to test all other factor X analogs for the properties according to the present invention.

Example 3:

a. Activity of the rFX/FXIa (Q-R/I) analog compared to rFX wild type in FIX-deficient plasma

To test the functionality of the rFX analog, FIX-deficient plasma is mixed with the supernatant of the CHO-rFX/FXIa (Q-R/I) cell culture, and the coagulation time cascade is measured after the intrinsic coagulation cascade has been activated (Table 1). For this purpose, 50 µL of the supernatant which was partially concentrated by ultrafiltration, 50 µL of FIX-deficient plasma, and 50 µL of DAPPTIN (Baxter AG) were mixed. After an incubation time of 4 min at 37°C, the reaction is initiated with 50 µL of 25 mM $CaCl_2$ which had been preheated to 37°C. The coagulation time is measured in the coagulometer (Amelung, KC10A). The activity in mU FIX is determined using a straight calibration line that is generated with plasmatic FIX. The coagulation times obtained with the rFX analogs are correspondingly given as mU FIX equivalents. Supernatants of the cell culture of rFX wild type-expressing CHO cells (CHO-rFX) and supernatants of the cell culture of nontransfected CHO cells (CHO neg.) serve as the control. To exclude nonspecific effects and to take into account experimental variations that are based on the conditions of the cell culture, 7 to 10 different cell culture surpernants of

cells, which have the same growth stages and similar expression rates, were tested for each construct.

Supernatants of cell cultures of CHO-rFX and CHO neg. lead to coagulation times similar to those of the dilution buffer in which the standards and samples are diluted and are thus given as `<1.56′ mU FIX equivalent (smallest evaluable value in the coagulation tests due to the straight calibration line).

Compared to the coagulation times that had been determined with 200 mU plasmatic FIX, however, the coagulation times for the supernatants of CHO-rFX/FXIa (Q-R/I) cells determined with concentrations of the analog in a range from 4.1 to 7.7 µg/mL were significantly shorter and are therefore given as `>200′ mU FIX equivalent.

These results show that by replacing the 8 C-terminal amino acids of the FX activation peptide with the 8 C-terminal amino acids of the activation peptide of factor IX, an rFX analog molecule was generated which, after activation of the intrinsic coagulation cascade, leads to a significant coagulation of a factor IX-deficient plasma.

b. Determination of the functional activity of the rFX/FXIa (Q-R/I) analog in FIX- and FVIII-deficient plasmas after a pretreatment of the supernatants of the cell culture with serine protease inhibitors.

To be able to eliminate the possibility that the coagulation times obtained with the rFX/FXIa (Q-R/I) analogs are not the result of the presence of traces of already activated rFX molecules in the supernatants of the cell culture but are instead the result of the conversion of the rFX analogs into rFXa after the activation of the intrinsic coagulation cascade by DAPPTIN, prior to use, all supernatants are mixed with a serine protease inhibitor (1 mM Pefabloc, Boehringer Mannheim) which permanently inactivates activated serine proteases. Excess inhibitor which would inhibit the coagulation following DAPPTIN activation is subsequently removed by means of dialysis against Tris pH 7.4, NaCl 50 mM, Tween 0.01%. The supernatants thus treated are subsequently used in the coagulation test.

The serine protease inhibitor concentration was selected to ensure that the quantities of FXa which lead to a coagulation similar to that of the untreated

rFX/FXIa (Q-R/I) cell culture supernatants were completely inhibited (Table 2). The results of this preliminary experiments show that an inhibitor concentration of 1 mM is able to completely inactivate FXa quantities which have considerably shorter coagulation times in FIX-deficient plasma than those obtained with the rFX/FXIa (Q-R/I) analogs (comparison with Table 1).

The functionality of the thus pretreated supernatants is measured both in the FIX and in the FVIII coagulation test (Table 3). The FVIII coagulation test is carried out in the same way as the FIX coagulation test, except that instead of FIX-deficient plasma, FVIII-deficient plasma is used, that the incubation prior to the initiation with $CaCl_2$ lasts 3 min, and that the calibration curve is plotted with plasmatic FVIII.

Supernatants of CHO-rFX cells are below the limit of evaluation (1.56 mU) both in FIX- and in FVIII-deficient plasma. Similarly, CHO neg. supernatant does not lead to a shorter coagulation time even if this supernatant was treated with 1 mU FXa prior to the treatment with the inhibitor. These controls show that even if already preactivated FX would be inactivated by the inhibitor in the cell culture supernatant ..., and that the coagulation is not mediated by potentially existing CHO-specific proteases.

The pretreated plasmatic FIX (FIX) in comparison with FIX that has not been pretreated (FIX without inhibitor and dialysis) has a lower coagulation activity in FIX-deficient plasma, which indicates that either a portion of the serine proteases, even in the unactivated form, is inhibited by the inhibitor or is lost as a result of the process (dialysis).

In spite of the pretreatment of the inhibitor, in FIX and in FVIII coagulation tests, no significant changes in the measured values are observed in the CHO-rFX/FXIa (Q-R/I) supernatants when compared to untreated supernatants. These experiments show that after activation of the intrinsic coagulation cascade, recombinant FX analog molecules which have a FIX activation cleavage site for FXIa lead to a significant coagulation of FIX- and FVIII-deficient plasmas.

In summary, it has been demonstrated that these recombinant FX/FXIa (Q-R/I) analog molecules have properties which indicate that the molecules may prove to be successful candidates for the production of therapeutic preparations which might be

useful in the treatment of patients suffering from hemophilia or from hemophilia with inhibitory antibodies.

Table 1: Functional activity of the rFX/FXIa (Q-R/I) molecules in FIX-deficient plasma.

Sample	Antigen µg\ml	mU FIX equivalent	Coagulation time in seconds
rFX/FXIa (Q-R/I)			
sup1	6.3	>200	46.5
sup2	4.4	>200	47.7
sup3	6.6	>200	46.4
sup4	4.8	>200	43.2
sup5	4.1	>200	45.2
sup6	6.4	>200	43.1
sup7	6.6	>200	43.1
sup8	4.3	>200	45.2
sup9	5.7	>200	39.4
sup10	7.7	>200	44.2
rFXwt			
sup1	2.1	<1.56	107.8
sup2	3.1	<1.56	97.8
sup3	1.6	3	84.7
sup4	2.4	<1.56	90.4
sup5	3.2	<1.56	92.4
sup6	4.4	<1.56	96.2
sup7	6.7	<1.56	92.7
CHO neg.		<1.56	179.7
Plasma FIX	1*		50.7
Plasma FIX	0.5*		54.5
Plasma FIX	0.125*		65.6
Plasma FIX	0.0625*		72.2
Plasma FIX	0.0078*		89.1
Buffer	0		109.7

200, 100, 25, 12.5 and 1.56 mU plasmatic FIX were used. To convert these values into µg/mL, it was assumed that 1 unit FIX corresponds to approximately 5 µg of FIX/mL.

Table 2: Determination of the inactivation of FXa by means of a preliminary treatment with Pefabloc and subsequent dialysis in the FIX coagulation test

Sample	mU used	Coagulation time in seconds	mU FIX equivalent
FXa	5	19.9	>200
FXa	1	32.5	>200
FXa	0.5	45	>200
FXa	0.1	76.1	10
FXa + Inh	5	93.4	1.94
FXa + Inh	1	115.9	<1.56
FXa + Inh	0.5	113.5	<1.56
FXa + Inh	0.1	115.3	<1.56
Plasma	200	52.9	
Plasma	1.56	95.7	
Buffer		114.7	

Table 3: Functional activity of CHO-rFX/FXIa (W-R/I) supernatants of a cell culture after a preliminary treatment with Pefabloc and dialysis in FIX- and FVIII-deficient coagulation tests.

Sample	Antigen µg\ml	mU FIX equivalent	mU FVIII equivalent
rFX/FXIa (Q-R/I)			
sup1	6.3	>200	>200
sup2	4.4	100	>200
sup3	6.6	>200	>200
sup4	4.8	167	>200
sup5	4.1	>200	>200
sup6	6.4	>200	>200
sup7	6.6	>200	>200
sup8	4.3	>200	>200
sup9	5.7	>200	>200
sup10	7.7	>200	>200
rFXwt			
sup1	2.1	<1.56	<1.56
sup2	3.1	<1.56	<1.56
sup3	1.6	<1.56	<1.56
sup4	2.4	<1.56	<1.56
sup5	3.2	<1.56	<1.56
sup6	4.4	<1.56	<1.56
sup7	6.7	<1.56	<1.56
CHO neg.		<1.56	<1.56
CHO neg. + Fxa		<1.56	<1.56
Plasma FIX (-Inh. –Dial.)*	25mU	25	
Plasma FIX	25mU	4	
Plasma FVIII (-Inh. –Dial.)*	50mU		48
Plasma FVIII (-Inh. –Dial.)*	3.125mU		3

These samples were used in the tests without being treated (without the addition of serine protease inhibitor and dialysis).

Example 4:

Activity of purified rfX/fXIa(Q-R/I) molecules in vitro and in vivo

rfX/fXIa(Q-R/I) molecules were purified from cell culture supernatants of stable CHO-rfX/fXIa(Q-R/I) cell clones (established as described in example 1.c) by anion exchange chromatography. Because of the presence of partially proteolytically incompletely matured precursor rfX molecules in the supernatant of recombinant cell lines used for purification (figure 5, lane 8), the supernatant was first pre-incubated for 4 hours at 37°C with conditioned medium from CHO cell clones expressing a secreted form of the endoprotease furin. Furin is a serine protease which performs the conversion of fX single chain into heavy/light chain as well as the propeptide removal in vitro. By this treatment processing of immature fX molecules is completed in vitro (figure 5, lane 2 versus 8) avoiding the co-purification of inactive immature fX molecules. After addition of 10mM EDTA, 0,1% of Tween 80, 0,1mM Pefabloc and adjustment of the pH to 7,4, the supernatant was loaded onto a Fractogel EMD TMAE 650 (M) column equilibrated with Buffer A (20mM Tris, 120mM NaCl, 10mM EDTA, 0,1% Tween 80, pH7,4). The column was washed with 20mM Tris, 180mM NaCl, 0,1% Tween 80, pH 7,4. rfX Molecules were eluted with Buffer C (20mM Tris, 150mM NaCL, 10mM CaCl2, 0,1% Tween, pH 7,4). The Western Blot analysis of the different purification fractions (figure 5) shows that almost all rfX/fXI(Q-R/I) molecules of the elution fraction are present in form of the mature double chain (HC, LC; lane 6), similarly to purified plasma fX (lane7). In order to inhibit residual amounts of rfXa potentially present, which could have been formed during cell culture or the purification procedure, the elution fraction was subsequently treated with 10µM ERGck (Hematological Technologic Inc.), a specific fXa inhibitor. Excess of this inhibitor was removed by the subsequent diafiltration step with 10mM Tris, 8g/L NaCl, 4g/L NaCitrate, 0,01% Tween 80, pH7. The preparation was stored at –80°C until use. The same purification procedure was used for the preparation of rfX wild-type employed as a control in the following experiments.

a. Determination of the functional activity of purified rfX/fXIa(Q-R/I) in human and murine fVIII- and fIX-deficient plasma

In order to confirm the functional activity of the purified rfX/fXIa(Q-R/I) molecules, the aPTT (Activated Partial Thromboplastin Time) in fVIII- and fIX-deficient plasma from human and murine origin was measured in the presence of the rfX molecules. 100µl of deficient plasma (immunodepleted human plasma, Baxter AG; plasma from fVIII or fIX knock-out mice) was mixed with 50µl of a 10µg/ml purified fX molecule preparation (see above), 150µl DAPPTIN (Baxter AG) and incubated for 3 min at 37°C. The coagulation reaction was initiated by 150µl 25mM CaCl2. The clotting time was estimated as described in example 3. The percentage of activity was determined by using a standard curve Log (% activity) versus Log (clotting time). For the standard curve, 100µl of 100%, 80% 50%, 25% or 12,5% human or murine reference plasma was mixed to 50µl of the fX buffer and 150µl DAPPTIN and incubated for 3min at 37°C. The initiation of coagulation and estimation of clotting time was performed as described above. The reference plasmas consist of normal human plasma (Baxter AG) or plasma pool from normal mice mixed at different ratios with the corresponding fVIII or fIX deficient plasmas. The clotting time as well as the percentage of activity compared to normal plasma are given in table 4. The results show that a significant reduction of the clotting time is mediated by purified rfX/fXIa(Q-R/I) in all deficient plasma tested either from human or murine origin. A normalization of the aPTT is even observed in human and murine fIX-deficient plasma and in murine fVIII-deficient plasma. In contrast to the rfX-analogue, rfX wt molecules at the corresponding concentrations do not mediate a significant reduction of the clotting time in these plasmas. Furthermore, the normalization of the aPTT in deficient mice plasma indicates that the rfX/fXIa(Q-R/I) molecules are able to interact with the corresponding murine coagulation proteins mediating the fVIII and fIX bypass activity observed in vitro in human plasma, a prerequisite for testing these molecules in vivo in a mouse animal model.

b. Functional activity of the rfX/fXIa(Q-R/I) variant in vivo

The functionality of rfX/fXIa(Q-R/I), as a molecule exhibiting a fVIII bypass activity, was tested in vivo by using fVIII knock-out mice (Table 5). The validity of the fVIII knock out mice as an animal model system for testing fVIII bypass

Table 4: Functional Activity of purified rfX/fXIa(Q-R/I) in human fVIII and fIX-deficient plasma and in plasma from fVIII and fIX Knock-Out mice

	Normal Plasma	FVIII-def. Plasma	FVIII-def. Plasma + rfX wt	FVIII-def. Plasma + rfX/fXIa(Q-R/I)	FIX-def. Plasma	RfIX-def. Plasma + rfXwt	FIX-def. Plasma + rfX/fXIa(Q-R/I)
Coagulation Time in sec.	52.3	138.5	112.7	52.5	153.7	117.5	48.6
% Activity in mouse plasma	100	-	3.7	97	-	0.1	166
Coagulation Time in sec.	47.5	143.9	118.3	56.3	161.6	117.9	48
% Activity in human plasma	100	-	2.4	77	-	0.5	99

activity was controlled by using the commercially available plasma derived FEIBA preparation (Baxter AG). As a negative control, a preparation of purified rfX wt was used. In order to compare the effect of rfX/fXIa(Q-R/I) and rfXwt the unit definition for the dose to be administered was based on the fX activity determined in PT (prothrombin time) assay. For the PT assay, 100µl of fX molecule preparation and 100µl fX-deficient plasma (Baxter AG) was incubated for 2min at 37°C. The coagulation was initiated by 100µl of Calcium/Thromboplastin mixture (Baxter AG). The clotting time was estimated as described in example 3. The fX activity in units was determined by using a standard curve established with a fX reference standard (Baxter AG). The preparations were administered intravenously under anesthesia at the indicated dose in U/kg (table 5). 30min after injection the bleeding was induced by cutting the tail at a distance of 1cm from its end. The survival rate of the animals after 48 hours serves as a parameter for functionality. The significant reduction of mortality observed for the animals treated with FEIBA, demonstrates that fVIII knock-out mice represent a suitable model system. None of the animals treated with 300U rfXwt /kg survived, a result to be expected from the in vitro findings. However, the application of rfX/fXIa(Q-R/I) at 300U/kg resulted in a significant improvement of the survival rate (60%). This result demonstrated that the rfX-analogue molecules exhibit a significant fVIII bypass activity in vivo, at the dosage used.

Table 5: Determination of the survival rate of fVIII knock-out mice treated with rfX/fXIa(Q-R/I) variant and rfX wt

	FEIBA 300U/kg	FEIBA 150U/kg	rfX/fXI(Q-R/I) 300U/kg	RfX wt 300U/kg
Survival	3/3	1/2	6/10	0/3
%Survival	100	50	60	0

In conclusion, the functional properties of the rfX/fXIa(Q-R/I) molecule described in these experiments, demonstrate that it represents a prime candidate for the

development of a alternate therapeutic agent for the treatment of hemophiliacs having developed inhibitory antibodies.

Claims

1. A factor X analog which contains a modification between Glu226 and Ile235, relative to the amino acid numbering according to Figure 1.

2. The factor X analog of claim 1 wherein the modification is between Glu226 and Arg 234.

3. The factor X analog as claimed in Claim 1, characterized in that it contains a factor X sequence with Glu226-R8-R7-R6-R5-R4-R3-R2-Arg234-R1, wherein

a) R1 is an amino acid selected from the group Ile, Val, or Ala,

b) R2 is an amino acid selected from the group Thr, Ser, or Asn,

c) R3 is an amino acid selected from the group Phe, Leu, Arg, or Ile,

d) R4 is an amino acid selected from the group Asp. Lys, Thr, or Glu,

e) R5 is an amino acid selected from the group Asn, Ser, Lys, Met, Thr, or Asp;

f) R6 is an amino acid selected from the group Phe, Thr, Ser, Pro, Leu, or Ile,

g) R7 is an amino acid selected from the group Ser, Gln, Ile, Thr, Asn, or Pro, and

h) R8 is an amino acid selected from the group Gln, Ser, His, Tyr, or Glu.

4. The factor X analog of claim 1, characterized in that it contains a modification in the region of amino acids 227-233 of the factor X sequence, relative to the amino acid numbering according to Figure 1, as follows:

Gln227-Ser228-Phe229-Asn230-Asp231-Phe232-Thr233

5. The factor X analog of claim 4 wherein amino acid 235 is also modified.

6. The factor X analog of claim 1, characterized in that it contains a modification in the region of amino acids 227-233 of the factor X sequence, relative to the amino acid numbering according to Figure 1, as follows:

Ser227-Gln228-Th229-Ser230-Lys231-Leu232-Thr233.

7. The factor X analog of claim 6 wherein amino acid 235 is also modified.

8. The factor X analog of claim 1 wherein the modification forms a processing site for factor XIa or a derivative thereof.

9. The factor X analog of claim 1 characterized in that it has an additional modification in the region of the C-terminal factor X amino acid sequence.

10. The factor X analog as claimed in Claim 9, characterized in that it has a modification in the C-terminal region of the ß-peptide cleavage site.

11. The factor X analog of claim 1 wherein said modification permits an *in vivo* activation of the factor X analog into native factor Xa or a factor Xa analog.

12. The factor X analog of claim 1 wherein said modification permits an *in vitro* activation of factor X analog into native factor Xa or a factor Xa analog.

13. The factor X analog of claim 1 wherein said analog contains an intact ß-peptide.

14. The factor X analog of claim 1 in the form of a double-chain molecule.

15. The factor X analog of claim 1 having a shortened C-terminal region.

16. A recombinant DNA coding for the factor X analog of claim 1 contained in a vector for the recombinant expression of the coded protein.

17. A preparation containing a purified factor X analog or a precursor protein thereof, said factor X analog containing a modification between Glu226 and Ile235, relative to the amino acid numbering according to Figure 1.

18. The preparation of claim 17 wherein the modification is between Glu226 and Arg 234.

19. The preparation as claimed in Claim 17, characterized in that the modification forms a cleavage site for factor XIa or a derivative thereof.

20. The preparation of claim 17, characterized in that the factor X analog is present in the form of FXα.

21. The preparation of claim 17 wherein the factor X analog has a shortened C-terminal amino acid sequence.

22. The preparation of claim 17, characterized in that it contains factor X analog as a double-chain molecule.

23. The preparation of claim 17, characterized in that it contains a single-chain factor X analog in enzymatically inactive form, with a purity of a minimum of 80% and that it does not contain inactive proteolytic intermediates of factor X/Xa analog.

24. The preparation of claim 17, characterized in that it contains factor X analog as a single-chain molecule.

25. The preparation of claim 17, characterized in that it contains a factor X analog which has a modification that permits an *in vivo* activation of the factor X analog into native factor Xa or into a factor Xa analog.

26. The preparation of claim 17, characterized in that it contains a factor X analog which has a modification that permits an *in vitro* activation of the factor X analog into native factor Xa or into a factor Xa analog.

27. The preparation of claim 17, characterized in that it is formulated as a pharmaceutical preparation.

28. A preparation containing an activated factor X analog obtainable by activation of the factor X analog of claim 1, said activated factor X analog having high stability and structural integrity, said preparation being free from inactive factor X/Xa analog intermediates and autoproteolytic factor X decomposition products.

29. The preparation of claim 28, characterized in that it contains a physiologically acceptable matrix and is present in a form that is stable to storage.

30. The preparation of claim 28, characterized in that it contains a blood factor or an activated form of a blood factor as an additional component.

31. The preparation of claim 30, characterized in that it contains a minimum of one component with factor VIII inhibitory bypass activity as an additional component.

32. The preparation of claim 17, characterized in that it is formulated as a pharmaceutical compound and is present as a multi-component preparation.

33.The use of the preparation of claim 17 to produce a drug.

34. The use of the recombinant DNA of Claim 16 to produce a drug.

35. A method for the production of the preparation of claim 17, characterized in that the factor X analog which was obtained by means of recombinant production is isolated and purified by means of a chromatographic process.

36. A method for the production of preparation of a factor X analog, said method comprising the following steps:

- preparation of a recombinant DNA coding for the factor X analog of claim 1 contained in a vector for the recombinant expression of the coded protein
- transformation of a suitable cell
  - expression of the factor X analog
  - isolation of the factor X analog, and

- purification of the factor X analog by means of a chromatographic process.

37. The method as claimed in claim 36, characterized in that after expression of the factor X analog it is activated by factor XIa or a derivative thereof.

38. The method as claimed in claim 35, characterized in that the factor X analog is isolated in the form of a double-chain molecule.

39. The method of claim 35, characterized in that the double-chain factor X analog is cleaved with factor XIa or a derivative thereof.

40. The method of claim 35, characterized in that the factor X analog is isolated in the form of a single-chain molecule.

41. The method as claimed in Claim 39, characterized in that a single-chain, factor X analog is processed with furin or a derivative thereof and that further allows the activation with factor XIa or a derivative thereof into factor Xa or the factor Xa analog.

42. A method for the production of a preparation containing activated factor Xa or a factor Xa analog, characterized by the fact that a factor X analog which was produced using the method of claim 35 is subjected to an activation step.

43. A method as claimed in Claim 35, characterized by the fact that a purified factor Xa analog or a native factor Xa with a high stability and structural integrity which is free from inactive factor X/Xa intermediates is obtained.

Abstract

This invention describes a factor Xa analog which has a substitution of a minimum of one of the amino acid between Glu226 and Arg234 and possibly Ile235, relative to the amino acid numbering according to Figure 1, a preparation containing the activated form of the factor X analog, and a method for the production of these molecules.

(-40)
1
Met Gly Arg Pro Leu His Leu Val Leu Leu Ser Ala Ser Leu Ala Gly Leu Leu Leu
ATG GGG CGC CCA CTG CAC CTC GTC CTG CTC AGT GCC TCC CTG GCT GGC CTC CTG CTG
9 18 27 36 45 54

(-4) (-1)
40
Leu Gly Glu Ser Leu Phe Ile Arg Arg Glu Gln Ala Asn Asn Ile Leu Ala Arg Val Thr Arg
CTC GGG GAA AGT CTG TTC ATC CGC AGG GAG CAG GCC AAC AAC ATC CTG GCG AGG GTC ACG AGG
66 75 84 93 102 111 120

(+1)
41
Ala Asn Ser Phe Leu Glu Glu Met Lys Lys Gly His Leu Glu Arg Glu Cys Met Glu Glu Thr
GCC AAT TCC TTT CTT GAA GAG ATG AAG AAA GGA CAC CTC GAA AGA GAG TGC ATG GAA GAG ACC
129 138 147 156 165 174 183

Cys Ser Tyr Glu Glu Ala Arg Glu Val Phe Glu Asp Ser Asp Lys Thr Asn Glu Phe Trp Asn
TGC TCA TAC GAA GAG GCC CGC GAG GTC TTT GAG GAC AGC GAC AAG ACG AAT GAA TTC TGG AAT
192 201 210 219 228 237 246

Lys Tyr Lys Asp Gly Asp Gln Cys Glu Thr Ser Pro Cys Gln Asn Gln Gly Lys Cys Lys Asp
AAA TAC AAA GAT GGC GAC CAG TGT GAG ACC AGT CCT TGC CAG AAC CAG GGC AAA TGT AAA GAC
255 264 273 282 291 300 309

Gly Leu Gly Glu Tyr Thr Cys Thr Cys Leu Glu Gly Phe Glu Gly Lys Asn Cys Glu Leu Phe
GGC CTC GGG GAA TAC ACC TGC ACC TGT TTA GAA GGA TTC GAA GGC AAA AAC TGT GAA TTA TTC
318 327 336 345 354 363 372

Thr Arg Lys Leu Cys Ser Leu Asp Asn Gly Asp Cys Asp Gln Phe Cys His Glu Glu Gln Asn
ACA CGG AAG CTC TGC AGC CTG GAC AAC GGG GAC TGT GAC CAG TTC TGC CAC GAG GAA CAG AAC
381 390 399 408 417 426 435

Ser Val Val Cys Ser Cys Ala Arg Gly Tyr Thr Leu Ala Asp Asn Gly Lys Ala Cys Ile Pro
TCT GTG GTG TGC TCC TGC GCC CGC GGG TAC ACC CTG GCT GAC AAC GGC AAG GCC TGC ATT CCC
444 453 462 471 480 489 498

178 179 180 181 182 183
Thr Gly Pro Tyr Pro Cys Gly Lys Gln Thr Leu Glu Arg Arg Lys Arg Ser Val Ala Gln Ala
ACA GGG CCC TAC CCC TGT GGG AAA CAG ACC CTG GAA CGC AGG AAG AGG TCA GTG GCC CAG GCC
507 516 525 534 543 552 561

Thr Ser Ser Ser Gly Glu Ala Pro Asp Ser Ile Thr Trp Lys Pro Tyr Asp Ala Ala Asp Leu
ACC AGC AGC AGC GGG GAG GCC CCT GAC AGC ATC ACA TGG AAG CCA TAT GAT GCA GCC GAC CTG
570 579 588 597 606 615 624

R6
229
Asp Pro Thr Glu Asn Pro Phe Asp Leu Leu Asp Phe Asn Gln Thr Gln Pro Glu Arg Gly Asp
GAC CCC ACC GAG AAC CCC TTC GAC CTG CTT GAC TTC AAC CAG ACG CAG CCT GAG AGG GGC GAC
633 642 651 660 669 678 687

R5 R4 R3 R2 R1
234 235
Asn Asn Leu Thr Arg Ile Val Gly Gly Gln Glu Cys Lys Asp Gly Glu Cys Pro Trp Gln Ala
AAC AAC CTC ACC AGG ATC GTG GGA GGC CAG GAA TGC AAG GAC GGG GAG TGT CCC TGG CAG GCC
696 705 714 723 732 741 750

Fig. 1-1

```
Leu Leu Ile Asn Glu Glu Asn Glu Gly Phe Cys Gly Gly Thr Ile Leu Ser Glu Phe Tyr Ile
CTG CTC ATC AAT GAG GAA AAC GAG GGT TTC TGT GGT GGA ACT ATT CTG AGC GAG TTC TAC ATC
        759         768         777         786         795         804         813

Leu Thr Ala Ala His Cys Leu Tyr Gln Ala Lys Arg Phe Lys Val Arg Val Gly Asp Arg Asn
CTA ACG GCA GCC CAC TGT CTC TAC CAA GCC AAG AGA TTC AAG GTG AGG GTA GGG GAC CGG AAC
        822         831         840         849         858         867         876

Thr Glu Gln Glu Glu Gly Gly Glu Ala Val His Glu Val Glu Val Val Ile Lys His Asn Arg
ACG GAG CAG GAG GAG GGC GGT GAG GCG GTG CAC GAG GTG GAG GTG GTC ATC AAG CAC AAC CGG
        885         894         903         912         921         930         939

Phe Thr Lys Glu Thr Tyr Asp Phe Asp Ile Ala Val Leu Arg Leu Lys Thr Pro Ile Thr Phe
TTC ACA AAG GAG ACC TAT GAC TTC GAC ATC GCC GTG CTC CGG CTC AAG ACC CCC ATC ACC TTC
        948         957         966         975         984         993        1002

Arg Met Asn Val Ala Pro Ala Cys Leu Pro Glu Arg Asp Trp Ala Glu Ser Thr Leu Met Thr
CGC ATG AAC GTG GCG CCT GCC TGC CTC CCC GAG CGT GAC TGG GCC GAG TCC ACG CTG ATG ACG
       1011        1020        1029        1038        1047        1056        1065

Gln Lys Thr Gly Ile Val Ser Gly Phe Gly Arg Thr His Glu Lys Gly Arg Gln Ser Thr Arg
CAG AAG ACG GGG ATT GTG AGC GGC TTC GGG CGC ACC CAC GAG AAG GGC CGG CAG TCC ACC AGG
       1074        1083        1092        1101        1110        1119        1128

Leu Lys Met Leu Glu Val Pro Tyr Val Asp Arg Asn Ser Cys Lys Leu Ser Ser Ser Phe Ile
CTC AAG ATG CTG GAG GTG CCC TAC GTG GAC CGC AAC AGC TGC AAG CTG TCC AGC AGC TTC ATC
       1137        1146        1155        1164        1173        1182        1191

Ile Thr Gln Asn Met Phe Cys Ala Gly Tyr Asp Thr Lys Gln Glu Asp Ala Cys Gln Gly Asp
ATC ACC CAG AAC ATG TTC TGT GCC GGC TAC GAC ACC AAG CAG GAG GAT GCC TGC CAG GGG GAC
       1200        1209        1218        1227        1236        1245        1254

Ser Gly Gly Pro His Val Thr Arg Phe Lys Asp Thr Tyr Phe Val Thr Gly Ile Val Ser Trp
AGC GGG GGC CCG CAC GTC ACC CGC TTC AAG GAC ACC TAC TTC GTG ACA GGC ATC GTC AGC TGG
       1263        1272        1281        1290        1299        1308        1317

Gly Glu Ser Cys Ala Arg Lys Gly Lys Tyr Gly Ile Tyr Thr Lys Val Thr Ala Phe Leu Lys
GGA GAG AGC TGT GCC CGT AAG GGG AAG TAC GGG ATC TAC ACC AAG GTC ACC GCC TTC CTC AAG
       1326        1335        1344        1353        1362        1371        1380

                                   469 470                 475 476             480
Trp Ile Asp Arg Ser Met Lys Thr Arg Gly Leu Pro Lys Ala Lys Ser His Ala Pro Glu Val
TGG ATC GAC AGG TCC ATG AAA ACC AGG GGC TTG CCC AAG GCC AAG AGC CAT GCC CCG GAG GTC
       1389        1398        1407        1416        1425        1434        1443

                          488
Ile Thr Ser Ser Pro Leu Lys TER
ATA ACG TCC TCT CCA TTA AAG TGA
       1452        1461    1467
```

Pre-/Propeptid
'Connecting' Tripeptide
Activation Peptide

Fig. 1-2

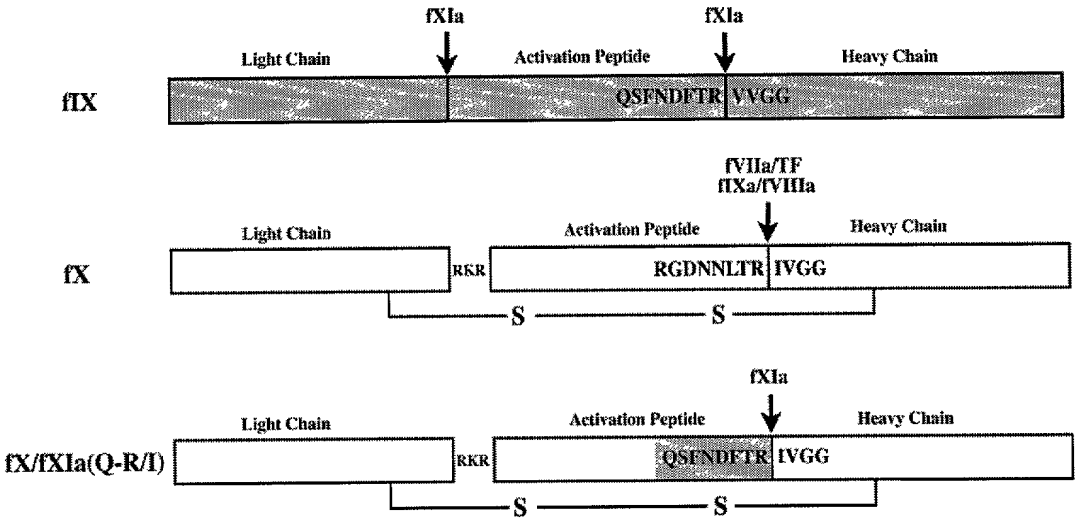
fIX
fXIa
fXIa
Light Chain
Activation Peptide
Heavy Chain
QSFNDETR
VVGG
fX
fVIIa/TF
fIXa/fVIIIa
Light Chain
Activation Peptide
Heavy Chain
RKR
RGDNNLTR
IVGG
S
S
fX/fXIa(Q-R/I)
fXIa
Light Chain
Activation Peptide
Heavy Chain
RKR
QSFNDETR
IVGG
S
S

Fig. 2

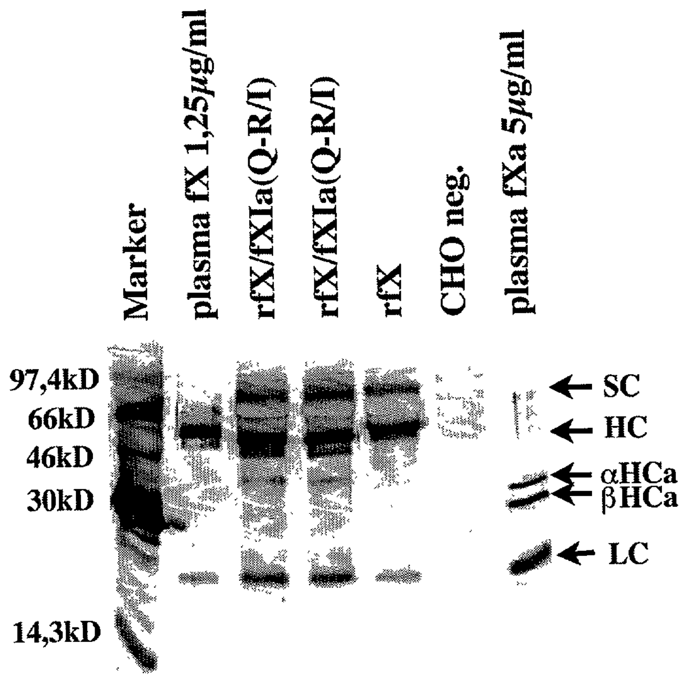
Marker
plasma fX 1,25μg/ml
rfX/fXIa(Q-R/I)
rfX/fXIa(Q-R/I)
rfX
CHO neg.
plasma fXa 5μg/ml
97,4kD
66kD
46kD
30kD
14,3kD
SC
HC
αHCa
βHCa
LC

Fig. 3

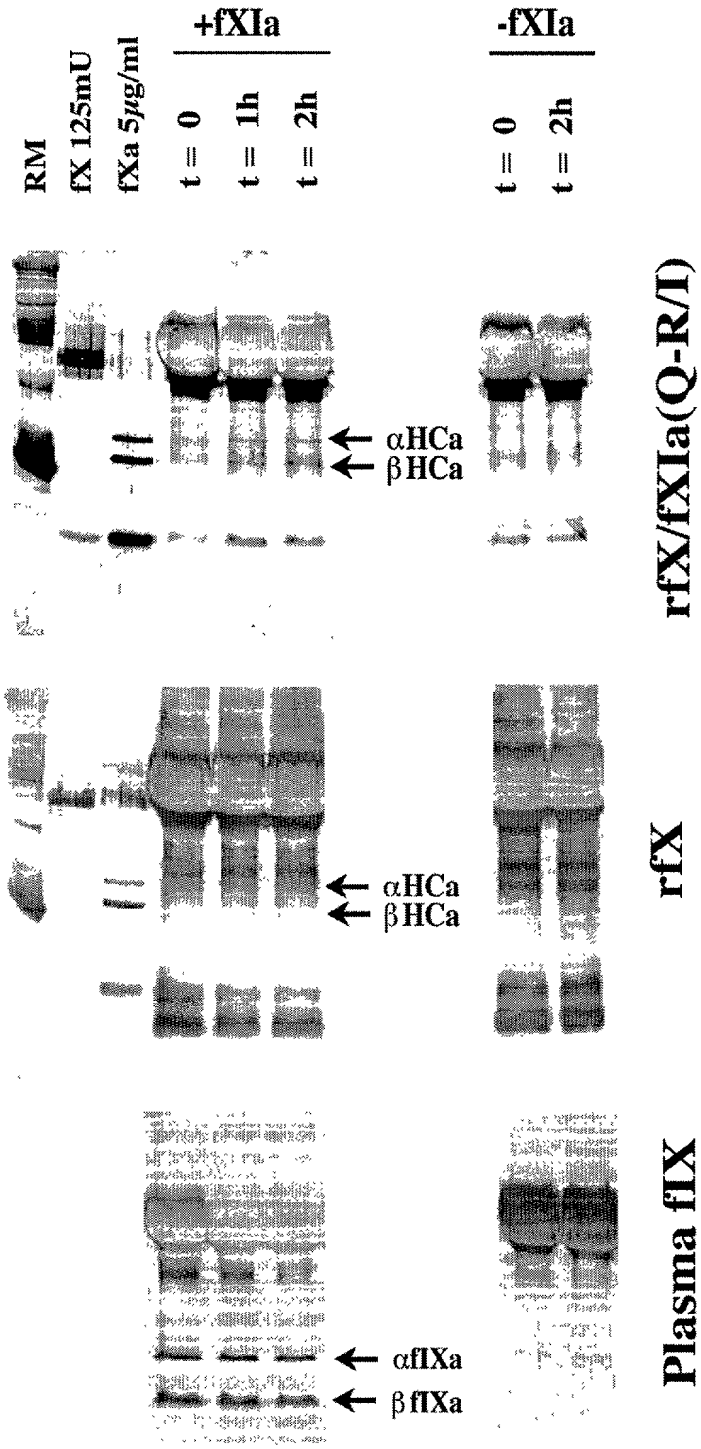
+fXIa
-fXIa
RM
fX 125mU
fXa 5μg/ml
t = 0
t = 1h
t = 2h
t = 0
t = 2h
αHCa
βHCa
rfX/fXIa(Q-R/I)
αHCa
βHCa
rfX
αfIXa
βfIXa
Plasma fIX

Fig. 4

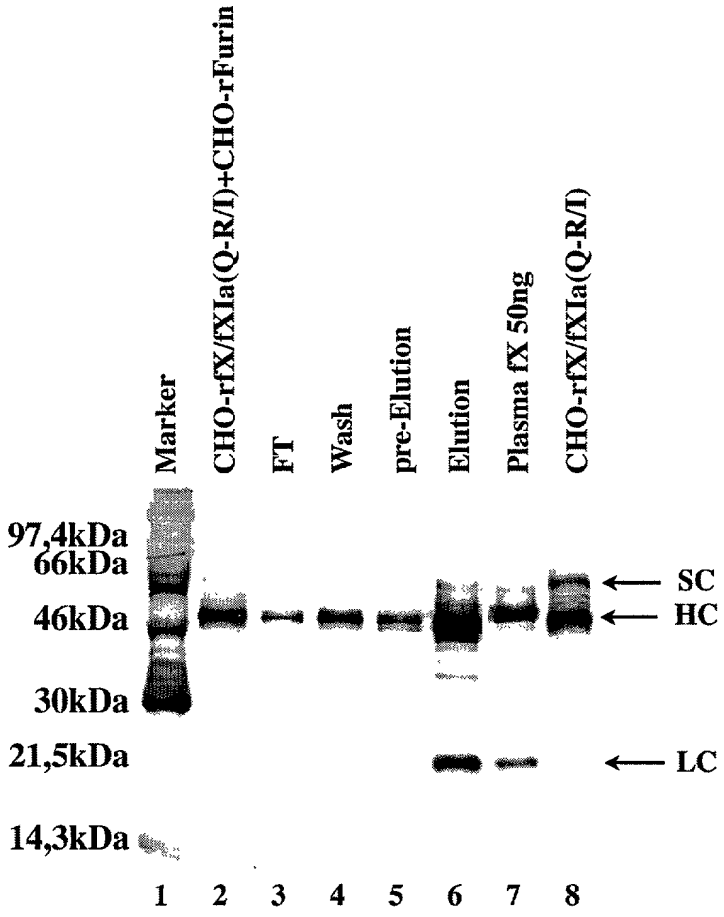
Marker
CHO-rFX/FXIa(Q-R/I)+CHO-rFurin
FT
Wash
pre-Elution
Elution
Plasma fX 50ng
CHO-rFX/FXIa(Q-R/I)
97,4kDa
66kDa
46kDa
30kDa
21,5kDa
14,3kDa
SC
HC
LC
1 2 3 4 5 6 7 8

Fig. 5